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The Isomer Ratios of Urinary Coproporphyrins I – IV are pH-Dependent[1])

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Summary: The percentage of porphyrinogens as related to total porphyrin excretion was determined in the urine of healthy subjects. Acidic urines (pH 5.0 to 5.9) contained 62.9 ± 10.7% ($\bar{x} \pm s$, N = 11) porphyrinogens, whereas in neutral urines (pH 6.0 to 7.2) a somewhat lower percentage (51.2 ± 15.3%, N = 11) was detected. However, there was no significant difference between the mean porphyrinogen contents of acidic and neutral urines.

Evidence was found for a previously unreported pH-dependent influence on the isomer ratios of urinary coproporphyrins I and III. Acidic urines (N = 18) from healthy subjects showed significantly higher percentages of isomer I (27.1 ± 6.4%), isomer II (2.7 ± 1.1%), and isomer IV (5.0 ± 1.3%) as compared to respective values from neutral urines (22.2 ± 5.1% isomer I, 0.6 ± 0.6% isomer II, and 1.5 ± 1.3% isomer IV; N = 16, $p < 0.001$). Conversely, the percentage of isomer III was markedly lower in acidic urines than in neutral urines (65.1 ± 7.9% vs. 75.9 ± 5.4%; $p < 0.001$). The same relationship was confirmed in an individual subject by analysis of a series of urines (N = 13) with pH values ranging from 5.4 to 7.3. These results point to the possibility that the atypical coproporphyrin isomers II and IV are predominantly formed by an increased isomerization rate of coproporphyrinogens under acidic intravesical conditions.

Introduction

Coproporphyrins of the series I and III are the principal isomers to be excreted in human urine, whereas the atypical isomers II and IV are only present in small amounts (1–3). It has been suggested that formation of the latter results from non-enzymatic self-isomerization of the naturally occurring coproporphyrinogens (3). Porphyrinogens are the metabolically active intermediates in the biosynthesis of haem. They are easily oxidized to the corresponding porphyrins in vivo and in vitro. Both porphyrinogens and porphyrins are excreted in urine (4). The proportion of porphyrins and their reduced precursors has been determined by various methods in urine of both porphyric patients (5–7) and healthy controls (7).

In the course of systematic investigations on the formation of the atypical coproporphyrins II and IV by means of isocratic ion-pair high-performance liquid chromatography (HPLC), we previously observed a stringent influence of urinary pH on the respective isomer ratios (8).

Here we report our subsequent studies on the influence of urinary pH on porphyrinogen excretion and on the isomer ratios of the individual coproporphyrins I–IV.

Materials and Methods

Individuals investigated

Spontaneous urine samples of apparently healthy persons of the laboratory staff were used in the study. One of us (P. L.) took voluntarily part in the enforced pH shifting experiment. Acidic urines were obtained by a high intake of ascorbic acid (up to 5 g per day), while neutral and alkaline urines were produced after ingestion of large doses of sodium-potassium-hydrogen citrate (up to 10 g per day).

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Determination of the porphyrinogen excretion in urine

A 2.0-ml urine sample was applied to a disposable anion-exchange column (AG1-X8, 50–100 mesh, BIO-RAD, Munich, Germany) shortly after excretion. Interfering substances were removed with water. The porphyrins were eluted with 4 ml 3 mol/l HCl and quantitated by recording the second derivative spectra of the *Soret* band in the wavelength range between 450 and 350 nm (9).

Another 2.0-ml sample of the same urine specimen was oxidized for 10 min at room temperature with a solution of 2 mg I_2 and 4 mg KI in 2 ml water. The surplus of iodine was removed with $Na_2S_2O_3$. Total porphyrins were isolated and quantified as described above. To calculate the relative porphyrinogen excretion, the increase of peak height after oxidation was divided by the peak height of the corresponding total porphyrins.

Preparation of urine samples for isomer analysis

Urine samples (50 ml, pH 5–7) were oxidized with a solution of 50 mg I_2 and 100 mg KI in 10 ml water immediately after excretion. The mixture was allowed to react 10 min at room temperature. Excess iodine was eliminated with $Na_2S_2O_3$, and subsequently 50 ml 3.7 mol/l H_3PO_4 were added. The porphyrins were adsorbed on Sep-Pak C_{18} cartridges (Waters, Eschborn, Germany) and eluted with methanol/acetone (1 + 1, by vol.). The solvents were removed under vacuum, and the residue was neutralized with aqueous sodium acetate, then adjusted to pH 3.5 with acetic acid. After adsorption on 200 mg talc (E. Merck, Darmstadt, Germany) the porphyrins were eluted with 10 ml methanol/H_2SO_4 (10 + 1, by vol.). The eluate was diluted tenfold with water and the porphyrins were adsorbed again on Sep-Pak cartridges as described. The isolated porphyrins were dissolved in 200 µl of 50 mmol/l methanolic tetrabutylammonium phosphate prior to HPLC analysis.

HPLC analysis

The simultaneous separation of the coproporphyrin isomers I–IV was achieved by isocratic ion-pair HPLC on a LiChrospher RP-18 column (2).

The proportions of the individual isomers were calculated from the respective peak area ratios.

Results and Discussion

Excretion of urinary porphyrinogens

A pH-dependent excretion of urinary porphyrinogens was investigated by analysing the porphyrinogen fraction of 22 freshly passed urine specimens. Urinary pH ranged from 5.0 to 7.2. Total porphyrinogens were determined by assaying the specimens *with* and *without* iodine oxidation.

The proportion of porphyrins occurring as porphyrinogens was somewhat higher in acidic urines (pH 5.0 to 5.9) in comparison with that in nearly neutral urines (pH 6.0 to 7.2) (tab. 1). The mean contents, however, did not differ significantly. We found porphyrinogen percentages between 23 and 76% (tab. 1). Similar amounts were present in the urine of porphyric patients (5, 6) and in healthy controls (7).

Tab. 1. Porphyrinogen content of 22 freshly passed urine specimens. Data are expressed as percentage of total porphyrin excretion.

Urine pH	N	$\bar{x} \pm s$ (%)	Range (%)
5.0–5.9	11	62.9 ± 10.7*	40–78
6.0–7.2	11	51.2 ± 15.3	23–76

* Not significantly different from the group with urine pH 6.0–7.2.

Preparation of urine samples for isomer analysis

An improved sample preparation method for the analysis of urinary coproporphyrin isomers I–IV was developed. First, porphyrinogens present in the freshly passed urine specimens were immediately oxidized to the corresponding porphyrins, because the latter are stable to further isomerization. Oxidation was performed with iodine at nearly neutral pH according to *Mauzerall* (10) in order to prevent chemical isomerization of porphyrinogens. Strongly acidic conditions for the oxidation step as reported by others (6, 7, 11) may lead to complete isomerization (10). Porphyrins were then extracted and efficiently purified by use of selective solid-phase sampling techniques with reversed-phase C_{18} materials and talc as sorbents. Subsequent isocratic ion-pair HPLC analysis produced clean chromatograms, with no detectable contaminants (fig. 1). The results were compar-

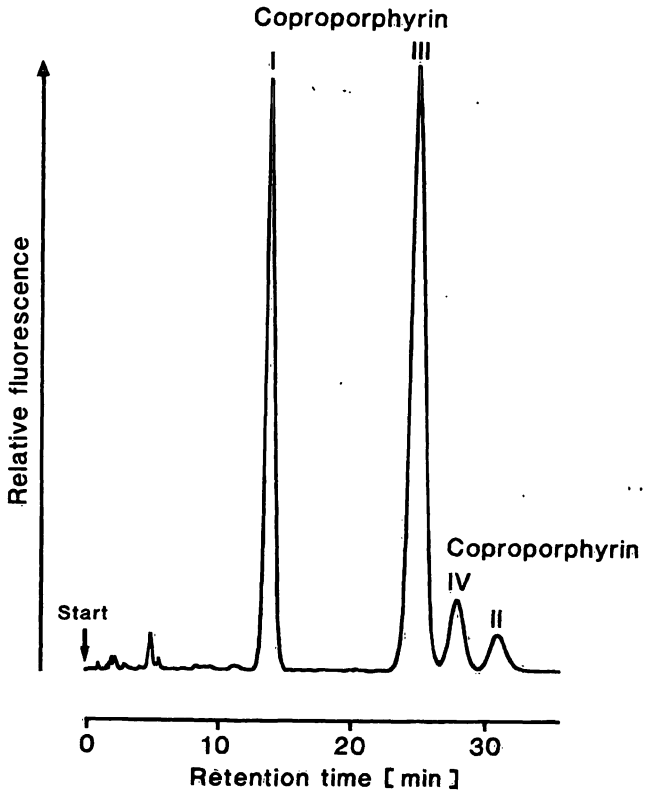

Fig. 1. HPLC separation of coproporphyrin isomers I–IV from an acidic urine (pH 5.3) of a healthy person after combined solid-phase purification steps.

able with those of the thin-layer chromatographic (TLC) purification method used in previous studies (2, 3). The advantage of the newly developed technique is a tenfold reduction of sample volume. This is achieved by omitting the TLC purification procedure, which requires additional preparation steps, e. g., extraction, esterification, and saponification of the respective porphyrin methyl esters. Due to the low porphyrin content of non-porphyric urines, sample volumes of 50 ml are necessary to obtain a complete isomer profile.

Relationship between coproporphyrin isomer ratios and urinary pH

The pH-dependence of coproporphyrin isomer ratios was determined in 34 freshly passed urine specimens from healthy subjects. Table 2 displays the results for the individual isomers in acidic urines (pH 5.0 to 6.0) and in nearly neutral urines (pH 6.1 to 7.6). The mean content of coproporphyrin III was approximately 10% lower in acidic urines compared with that in neutral urines ($p < 0.001$). On the other hand, acidic urines contained significantly higher levels of isomers I, II, and IV than neutral urines ($p < 0.001$). These effects were especially evident in the case of the atypical isomers II and IV. Here we found in acidic urines up to 5% of isomer II and 7% of isomer IV, whereas neutral urines contained only 0.1 to 0.3%.

To confirm these results, we demonstrated the arbitrary alteration of the respective isomer ratios by enforced shifting of the urinary pH value in an individual subject. Thus, from one person, we analysed 13 different urine specimens, with pH values between 5.4 and 7.3 (tab. 3). Under the experimental conditions applied, we observed the same relationship between coproporphyrin isomer ratios and urinary pH value as established for the whole study group (fig. 2a–d).

The strong influence of urine pH on the excretion rates of the individual coproporphyrin isomers can be explained by increased isomerization of coproporphyrinogens in vitro under relatively acidic conditions (3). In vivo, the predominantly occurring isomer III is non-enzymatically converted to the isomers I, II, and IV. This reaction is accelerated by acidic conditions. In neutral urines the isomerization rate is markedly reduced, and consequently higher proportions of isomer III are excreted combined with lower proportions of isomer I and only trace amounts of isomers II and IV. In addition, the isomerization rate is influenced by the retention time and temperature of urine in the human bladder. These factors were not standardized in our study, and therefore relatively wide

Tab. 2. Effect of urinary pH on the isomeric composition of coproporphyrins I–IV in 34 freshly passed urine specimens. Data are expressed as percentage of total urinary coproporphyrins.

Coproporphyrins	I	II	III	IV
Urine pH 5.0–6.0 (N = 18)				
Mean (%)	27.1*	2.7*	65.2*	5.0*
s (%)	6.4	1.1	7.9	1.3
Range (%)	18–39	1.1–5.0	51–75	3.0–7.2
Urine pH 6.1–7.6 (N = 16)				
Mean (%)	22.0	0.6	75.9	1.5
s (%)	5.1	0.6	5.4	1.3
Range (%)	12–31	0.1–2.5	67–87	0.1–5.2

* Significantly different ($p < 0.001$) from the group with urine pH 6.1–7.6.

Tab. 3. Effect of urinary pH on the isomeric composition of coproporphyrins I–IV in 13 freshly passed urine specimens from an individual healthy subject. Data are expressed as percentage of total urinary coproporphyrins.

Coproporphyrins	I	II	III	IV
Urine pH 5.4–6.0 (N = 6)				
Mean (%)	27.6	2.1	66.1	4.2
s (%)	2.2	0.6	3.7	1.6
Range (%)	24–30	1.6–3.4	60–72	2.7–6.7
Urine pH 6.1–7.3 (N = 7)				
Mean (%)	21.1	0.8	75.9	2.2
s (%)	3.0	0.5	2.3	1.2
Range (%)	17–25	0.2–1.9	73–80	0.3–4.1

ranges of the respective isomer ratios were found, particularly in the case of isomer I. In vitro kinetic experiments revealed a clearly time-dependent influence on the isomerization rate of coproporphyrinogen III. Thus, after 18 h at pH 5.2 and 37 °C, we observed an isomerization rate of 21% (corresponding to 5.6% isomer I, 5.2% isomer II, and 10.2% isomer IV) (unpublished results). After 24 h, under the same conditions, we found 29.8% isomerization (corresponding to 7.8% isomer I, 7.9% isomer II, and 14.1% isomer IV) (3).

In healthy subjects, the relative percentages of urinary coproporphyrins I and III are 25 and 75%, respectively (12). It is well known that these isomer ratios are considerably changed either by primary porphyrias, e. g., congenital erythropoietic porphyria, or by secondary coproporphyrinurias, e. g., in patients with certain types of liver diseases, *Dubin-Johnson* syndrome and *Rotor*'s syndrome (12). These deviations

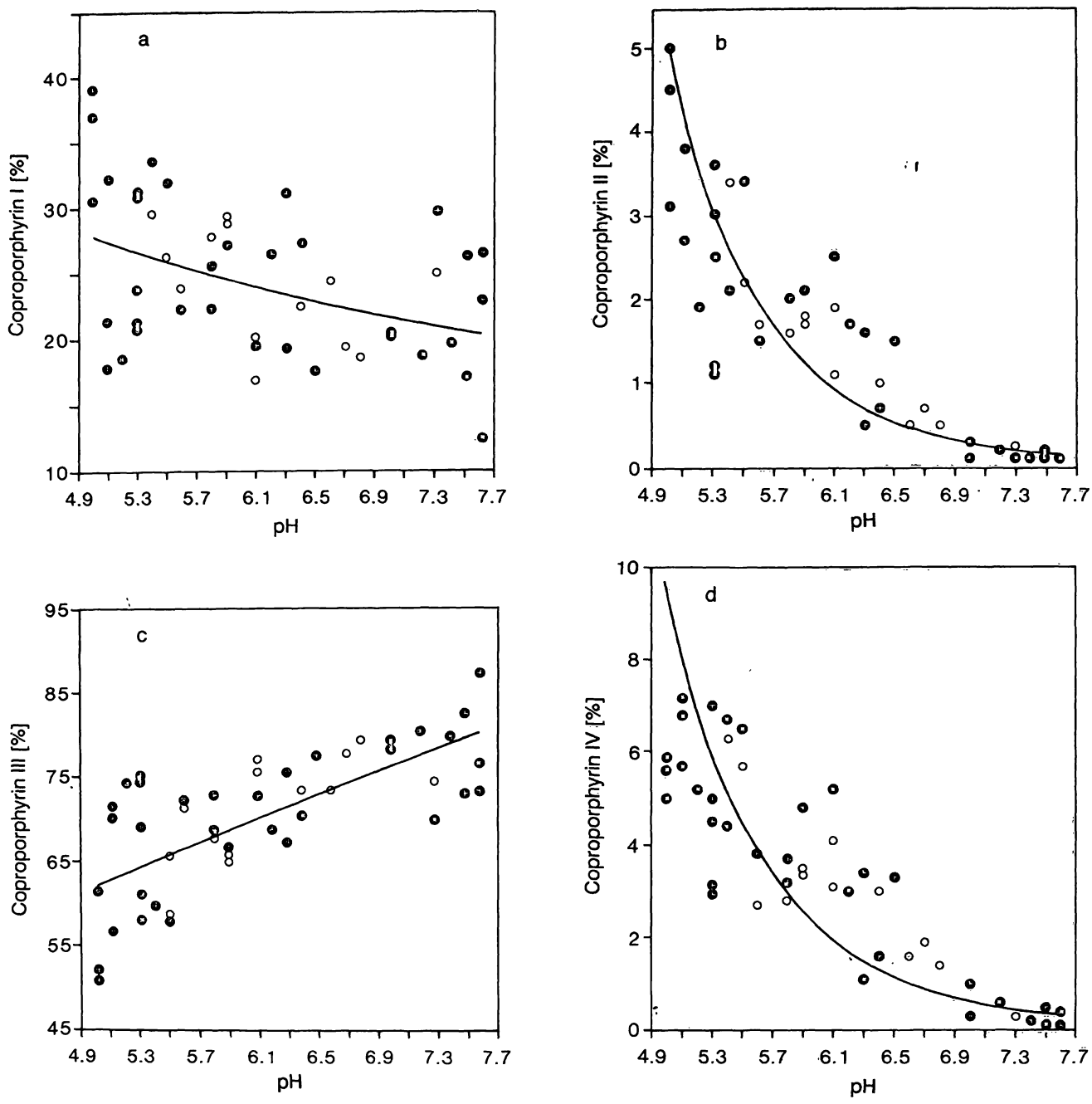

Fig. 2. Effect of urinary pH on the excretion of coproporphyrins (percentage of total coproporphyrins) in the urine of an individual subject (o) and the whole study group (●).
a = coproporphyrin I b = coproporphyrin II c = coprophorphyrin III d = coproporphyrin IV

are due to altered activities of enzymes involved in the biosynthesis of haem or by impaired coproporphyrin transport mechanisms (13). The methodology previously used for the estimation of urinary porphyrin isomer ratios allowed only the determination of isomers I and III. However, two independent HPLC techniques are available for the simultaneous separation of all four coproporphyrin isomers, namely the hydrophobic interaction technique (14) and the ion-pair technique (2, 15). Application of these advanced separation methods to the pH-related analysis of urinary coproporphyrins from patients with abnormal isomer distribution might be helpful for further elucidation of the underlying excretion mechanisms.

In conclusion, measurement of coproporphyrin isomers I—IV in urines of healthy subjects exhibited an unexpected influence of urinary pH on the excretion rates of the individual isomers. Alteration of the respective isomer ratios can be explained by non-enzymatic, intravesical isomerization of the naturally occurring coproporphyrinogens. It is suggested that the non-enzymatic change of side-chain sequence on the intact porphyrinogen molecule might be initiated by acid-catalysed ring-opening and rearrangement via spiro-cyclic intermediates (16).

Further studies, such as pH-related investigation of coproporphyrin isomer ratios in different clinical materials, will test the usefulness of the technique described here and may contribute to a better understanding of the metabolism of porphyrin isomers.

Acknowledgement

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